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CHAPTER 5

Summary and General Discussion

Manuel Schmitz

The work presented in this thesis originates from the working hypothesis that mechanical cues, acting on the unsegmented part of the paraxial mesoderm in the early vertebrate embryo, could predominantly instruct its segmentation into somites [1]. In this perspective, extracellular matrix molecules mechanically couple the segmenting mesoderm to its adjacent tissues, like neural tube or notochord, and transduce mechanical forces between tissues. Different growth rates of adjacent tissues would then lead to differential strain between them, which would ultimately result into a periodic strain pattern along the unsegmented PSM [1]. This pattern could instruct cells to epithelialize and split into somites. Initially, this hypothesis neglected several decades of theoretical and experimental work dedicated to the “clock-and-wavefront model”, which describes the patterning of the PSM as the results of an interplay between opposing morphogen gradients and their influence on a cellular oscillator, called the “segmentation clock”.

Nevertheless, we set out to identify the appropriate model system and to develop the experimental tools that would allow us to test our working hypothesis by manipulating the mechanical cues acting on the PSM. As described in the introduction, an unsuccessful deviation via zebra fish embryos led us to use chicken embryos as our model organism. Their availability, flat geometry and transparency during early developmental stages and a relative large number of somites make them a very important model system for somitogenesis [2]. However, most established *in vitro* culturing techniques for chicken embryos expose the ventral side of the embryo to the air, thereby making it prone to drying out [3], [4]. An environmental chamber, controlling temperature and humidity around the embryo, can compensate for this, but often comes along with spatial constrictions that were hardly compatible with our plans to exert mechanical micromanipulations on living embryos.

Through numerous trials with embryos explanted by the filter paper carrier technique [4], we found that early chick embryos develop normally, even if kept fully submerged in a simple culture medium, consisting only of Pannett-Compton saline and freshly harvested thin albumen (**CHAPTER 2**). We could successfully publish this new culturing technique as the “submerged filter paper sandwich”, as in our approach the embryo is stabilized between two layers of thick filter paper [5]. We could show that our technique

is compatible with high resolution conventional light microscopic time lapse imaging and microsurgical interventions, like micro bead implantation, and assumed that it holds great potential for high resolution live fluorescent imaging [5].

Subsequently, the “submerged filter paper sandwich” allowed us to design a dedicated experimental setup around this new culturing technique (**CHAPTER 3**). As a layer of light mineral oil on top to the culture medium prevents evaporation, we only needed to provide a constant temperature of 37.5°C around the embryos. This was achieved by using a temperature-controlled beaker with a glass bottom, allowing for sample illumination from below on an upright microscope. In close collaborations with the mechanical workshop of the VUmc, we designed and manufactured a frame around this beaker that holds two micro motors. Metals arms, screwed to these motors, reach into the culture medium and carry the filter paper sandwich with the embryo. As the long-working-distance, upright zoom microscope (Zeiss Axiozoom.V16) used in our experiments was equipped with a motorized x-y-stage, we could design the motorized metal arms to carry up to three embryos, thereby tripling the number of acquired time lapse movies. Filter paper sandwiches, clamped onto the metals arms, were cut perpendicularly to the head-to-tail axis of the embryos, so that the embryos formed the only bridges between the filter paper halves. To manipulate the mechanical cues acting on the paraxial mesoderm (somites and PSM), one of the metals arms was moved under computer control according to a standardized protocol. This protocol included two pull intervals, separated by a resting period to let damaged tissue heal. After the second pull interval, the motorized arm was not moved anymore. Thereby, embryos were kept under deformation, despite some relaxation of the embryonic tissue. The stretching led to severe deformation of the entire embryos, while their development progressed normally.

In our time lapse movies of stretched embryos we regularly observed that somites that had already bud off the PSM prior to or did so after the stretching, over the course of several hours, reorganized into two or more stable subunits. These we coined “daughter somites” for their morphological consistence with somite divisions observed in N-cadherin and Cadherin-11 knockout mice [6], [7]. We quantified the mechanical deformation of somites after stretching (measured by their aspect ratios), compared it to somites in non-stretched control embryos and could show that the degree of

deformation predicts very reliably if a somite will undergo daughter somite formation or not. This shows, that, at least under experimental condition, morphological somite formation is a highly mechanically determined process that can be guided by geometrical boundary conditions of the PSM.

It has to be remarked that we did not observe any obvious influence of mechanical stretching on the genetic patterning of the PSM, which precedes morphological somite formation. Neither the periodicity of somite formation nor the patterning of essential marker of the anterior or posterior somite halves (as determined by *in-situ* hybridizations) were impaired by the stretching. Our results confirm that the genetic segmentation of the PSM, as described in the “clock and wavefront model” and morphological somite formation, i.e. the actual organization of epithelializing cells at the anterior tip of the PSM into somites, have to be treated as two different, though not independent, processes. While the genetic segmentation was not obviously impacted by mechanical stretching in our experiments, we clearly showed a potential instructive role for mechanical cues on morphological somite formation. Therefore, our results represent a logical next step to the work of Dias *et al.*, 2014, which was published when we had just observed our first cases of daughter somite formation in stretched embryos. In their paper, the authors showed that somite-like structures can form ectopically under absence of any morphogen gradients or genetic oscillators [8]. They hypothesized that somite rosette formation from epithelializing mesodermal cells is a process of self-organization, guided only by cell packing constraints. With our experiments, we add to this the aspect that also geometrical constraints of a (mechanically) deformed PSM can instruct this self-organization.

Additionally, we could also identify a potential mechanism how the demand for additional epithelial border cells during daughter somite formation is met. High-resolution confocal fluorescence images indicated that the demand for additional border cells is satisfied by the recruitment of mesenchymal cells from the somitocoel into the existing epithelium. The mechanical deformation creates discontinuities in the apical actin cortices of stretched somites. At the resulting interfaces, mesenchymal cells from the somite core undergo mesenchymal-epithelial transitions (MET) and get integrated into the somitic epithelium. Similarly, it has been shown previously that the

development of normal somitic epithelia involves a continuous addition of cells from the somitocoel by accretion and egression [9]. Our observations indicate that, by adding mesenchymal cells to the epithelium via a similar cellular behaviour during stretching, the epithelium adapts to the changing environment. Under sufficient deformation, this leads to daughter-somite formation. Upon stretching, we additionally observed a strong ectopic expression of EphA4 in the somitocoels, which was not present in control embryos. During normal somitogenesis, cell-cell signalling between receptor EphA4 in the rostral part of somite S-I and its ligand ephrin B2 in the caudal half of somite S0 has two effects: it induces formation of the somite gap [10], but also establishes epithelialization at somite boundaries by initiating a columnar morphology and cell polarity via apical redistribution of β -catenin [11]. Therefore, this ectopic *EphA4* expression could indicate a stretched-induced MET of mesenchymal cells from the somitocoel. Interestingly, *EphA4* expression is not accompanied by an ectopic expression of its upstream initiator *cMesol*, suggesting an additional mechanosensitive pathway leading to *EphA4* expression upon stretching. A contact-induced MET mechanism, as suggested here for daughter-somite formation, could underlie general epithelial self-organization during development and homeostasis of epithelia under mechanical stress. In fact, it has been hypothesized recently and confirmed specifically for heart progenitor cells during early cardiogenesis, that purely mechanical cues can induce and guide mesenchymal-to-epithelial transitions [12], [13]. This cell behaviour could be mediated via epithelial membrane-based signalling [14], [15], for example on the level of Eph and ephrin binding.

In **CHAPTER 3**, our efforts to visualize the cellular behaviour at the interface between the somitic epithelium and mesenchymal somitocoel cells during daughter somite formation were limited to fixated, fluorescently labelled samples. This did not allow us to document *in vivo* the actual breaking of the apical actin cortices in stretched somites undergoing daughter somite formation and the incorporation of mesenchymal cells into the existing epithelium. Therefore, in **CHAPTER 4** we present essential steps towards a new protocol for live imaging of F-actin dynamics in chicken embryos cultured *in vitro*. To this end, we combined the “submerged filter paper sandwich” with SiR-actin, a recently introduced fluorogenic, cell permeable probe based on a synthetic derivative of

jasplakinolide, which is known to bind F-actin competitively with phalloidin [16], [17]. We established a simple protocol, in which the culture medium, surrounding the embryo at all times, functions as a reservoir for the SiR-actin dye. By reducing the percentage of thin albumen in the culture medium, we successfully minimized its blocking effect on the SiR-actin uptake into the embryonic tissue. Thereby, we were able to use only small volumes of SiR-actin stock solution, making the protocol very cost effective.

We could confirm that with SiR-actin a specific staining pattern, very similar to fluorescently labelled phalloidin, can be achieved in fixated chicken embryos. Preliminary time lapse movies of stretched embryos incubated with SiR-actin indicate that during daughter somite formation apical F-actin localizes in two discrete centers that first appear interconnected, but then gradually release from each other. This organization into two centers coincides with the appearance of the somitic furrow that indicates where the mother somite buds off the anterior tip of the PSM. We have not yet succeeded in acquiring time lapse images at confocal resolution, but are confident that small technical adjustments could reveal the organization of F-actin during somite and daughter somite formation.

In summary, our results indicate that chick somite formation is phenotypically plastic under changing biomechanical conditions. This supports the idea that, like temperature, light regime or salinity [18], mechanical forces could be an additional cue to the induction of different phenotypes of segmental patterning during embryonic development. To further explore if the observed plasticity of segmental patterning during somitogenesis can also translate into modified vertebral number in later embryonic development, we would need to adapt and develop protocols to transplant stretched embryos back into the host egg. Though in principle possible [19], this could prove technically challenging due to the severe deformation of the embryos. It may therefore be more promising to transplant daughter somites into host chick embryos in the egg and see how skeletal patterning might be influenced.

The experimental tools we developed and the results presented in this thesis can only be a starting point to the further exploration of how mechanical forces guide patterning

processes like somitogenesis. In fact, after the experiments presented in this thesis had been finished, the embryo stretcher, as presented in **CHAPTER 3**, was lend to the FMI Friedrich Miescher Institute for Biomedical Research in Basle, Switzerland, where it will help to explore the role of mechanical cues and their interplay with biomechanical signals during axial patterning in Hydra.

REFERENCES

- [1] L. Truskinovsky, G. Vitale, and T. H. Smit, "A mechanical perspective on vertebral segmentation," *Int. J. Eng. Sci.*, vol. 83, pp. 1–14, 2014.
- [2] O. Pourquié, "The chick embryo: a leading model in somitogenesis studies," *Mech. Dev.*, vol. 121, no. 9, pp. 1069–1079, Sep. 2004.
- [3] C. D. Stern and R. Bachvarova, "Early chick embryos in vitro.," *Int. J. Dev. Biol.*, vol. 41, pp. 379–387, 1997.
- [4] S. C. Chapman, J. Collignon, G. C. Schoenwolf, and A. Lumsden, "Improved method for chick whole-embryo culture using a filter paper carrier," *Dev. Dyn.*, vol. 220, pp. 284–289, 2001.
- [5] M. Schmitz, B. K. A. Nelemans, and T. H. Smit, "A Submerged Filter Paper Sandwich for Long-term Ex Ovo Time-lapse Imaging of Early Chick Embryos," no. 118, p. e54636, 2016.
- [6] K. Horikawa, G. Radice, M. Takeichi, and O. Chisaka, "Adhesive Subdivisions Intrinsic to the Epithelial Somites," *Situ*, vol. 215, pp. 182–189, 1999.
- [7] Y. Kimura *et al.*, "Cadherin-II expressed in association with mesenchymal morphogenesis in the head, somite, and limb bud of early mouse embryos.," *Developmental biology*, vol. 169, pp. 347–358, 1995.
- [8] A. S. Dias, I. de Almeida, J. M. Belmonte, J. A. Glazier, and C. D. Stern, "Somites Without a Clock," *Science (80-.)*, vol. 343, no. 6172, pp. 791–795, 2014.
- [9] G. G. Martins, P. Rifes, R. Amândio, G. Rodrigues, I. Palmeirim, and S. Thorsteinsdóttir, "Dynamic 3D cell rearrangements guided by a fibronectin matrix underlie somitogenesis.," *PLoS One*, vol. 4, no. 10, p. e7429, 2009.
- [10] T. Watanabe, Y. Sato, D. Saito, R. Tadokoro, and Y. Takahashi, "EphrinB2 coordinates the formation of a morphological boundary and cell epithelialization during somite segmentation.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 18, pp. 7467–72, May 2009.
- [11] A. Barrios, R. J. Poole, L. Durbin, C. Brennan, N. Holder, and S. W. Wilson, "Eph / Ephrin Signaling Regulates the Mesenchymal- to-Epithelial Transition of the Paraxial Mesoderm during Somite Morphogenesis," *Curr. Biol.*, vol. 13, pp. 1571–1582, 2003.
- [12] H. Y. Kim, T. R. Jackson, and L. A. Davidson, "On the role of mechanics in driving mesenchymal-to-epithelial transitions," *Semin. Cell Dev. Biol.*, vol. 67, pp. 113–122, 2017.
- [13] T. R. Jackson, H. Y. Kim, U. L. Balakrishnan, C. Stuckenholtz, and L. A. Davidson, "Spatiotemporally Controlled Mechanical Cues Drive Progenitor Mesenchymal-to-Epithelial Transition Enabling Proper Heart Formation and Function," *Curr. Biol.*, vol. 27, no. 9, pp. 1326–1335, 2017.
- [14] B. Baum and M. Georgiou, "Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling," *J. Cell Biol.*, vol. 192, no. 6, pp. 907–917, 2011.
- [15] K. Campbell, J. Casanova, and H. Skaer, "Mesenchymal-to-epithelial transition of intercalating cells in Drosophila renal tubules depends on polarity cues from epithelial neighbours.," *Mech. Dev.*, vol. 127, no. 7–8, pp. 345–57.
- [16] G. Lukinavičius *et al.*, "Fluorogenic probes for live-cell imaging of the cytoskeleton," *Nat. Methods*, vol. 11, no. 7, pp. 731–733, 2014.

- [17] M. R. Bubb, A. M. J. Senderowicz, E. A. Sausville, K. L. K. Duncan, and E. D. Korn, "Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin," *J. Biol. Chem.*, vol. 269, no. 21, pp. 14869–14871, 1994.
- [18] P. Tibblin, H. Berggren, O. Nordahl, P. Larsson, and A. Forsman, "Causes and consequences of intra-specific variation in vertebral number," *Sci. Rep.*, vol. 6, 2016.
- [19] H. Nagai, M. Sezaki, H. Nakamura, and G. Sheng, "Extending the limits of avian embryo culture with the modified Cornish pasty and whole-embryo transplantation methods," *Methods*, vol. 66, no. 3, pp. 441–446, 2014.